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The determination of gemcitabine and 2′-deoxycytidine in human plasma and tissue by APCI tandem mass spectrometry

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Abstract

A fast, sensitive and accurate method for the determination of gemcitabine (difluorodeoxycytidine; dFdC) and deoxycytidine (CdR) in human plasma/tissue was developed using LC–MS/MS techniques. Effectiveness of the method is illustrated with the analysis of plasma from a phase I trial of dFdC administered as a 24 h infusion. The method was developed using $^{15}N_3$ CdR as an internal standard across the concentration range of 1–500 ng/ml, using a cold alcohol-protein precipitation followed by desorption with freeze drying. Sample clean-up for LC–MS/MS analysis was performed by an innovative liquid/liquid back extraction with ethyl acetate and water. Chromatography was performed using a Chrompak-spherisorb-phenyl-column (3.1 mm × 200 mm, 5 μm) with a 50 mM formic acid: acetonitrile (9:1) mobile phase eluted at 1 ml/min. Extracted samples were observed to be stable for a minimum of 48 h after extraction when kept at 4 °C. Detection was performed using an atmospheric pressure chemical ionization (APCI) source and mass spectrometric positive multi-reaction-monitoring-mode (+MRM) for dFdC (264 *m/z*; 112 *m/z*), CdR (228 *m/z*; 112 *m/z*), and $^{15}N_3$ CdR (231 *m/z*; 115 *m/z*) at an ion voltage of +3500 V. The accuracy, precision and limit-of-quantitation (LOQ) were as follows: dFdC: 99.8%, ±7.9%, 19 nM; CdR: 100.0%, ±5.3%, 22 nM, linear range LOQ to 2 μM. During 24 h infusion dFdC levels were detected with no interference from either CdR or difluorodeoxyuridine (dFdU). CdR co-eluted with dFdC but selectivity demonstrated no "crosstalk" between the compounds. In conclusion the analytical assay was very sensitive, reliable and robust for the determination of plasma and tissue concentrations of dFdC and CdR.

Keywords: Gemcitabine; Deoxycytidine; Difluorodeoxycytidine; Mass spectrometry; HPLC

1. Introduction

Gemcitabine (difluorodeoxycytidine, dFdC) is a deoxycytidine (CdR) analogue (Fig. 1) that has shown chemotherapeutic activity alone and in combination against a variety of solid tumor types such as ovarian, non-small cell lung, pancreatic, bladder, and head/neck squamous cell carcinomas [1–5].

Further investigation of gemcitabine alone and in combination with other chemotherapeutics against other cancer types is an on-going and expanding field. This is especially true concerning protocols involving combination therapies with other chemotherapy compounds such as cisplatin [6], irinotecan, velcade [7], alimta [8], carboplatin [9] and radiation [10]. In addition, several schedules are being explored next to the standard 30-min infusion at 800–1250 mg/m^2 and the fixed dose rate infusion at 10 mg/m^2 per 10 min [11]. In a randomized phase II trial comparing the standard 30-min infusion with a fixed dose rate (FDR) infusion it has been shown that longer FDR infusion demonstrated a better overall patient survival based on pharmacokinetic data [12]. Also, it has been reported previously that the continuous infusion of gemcitabine over a 24-h period compared to the standard every 3-day bolus showed a significant increase in murine anti-tumor activity [13]. The 24-h infusion has also been explored for non-small cell lung cancer with comparable response data compared to the 30 min schedule [14], however, no pharmacokinetics were reported. Plasma concentrations were presumably below the detection limits of standard HPLC methods.

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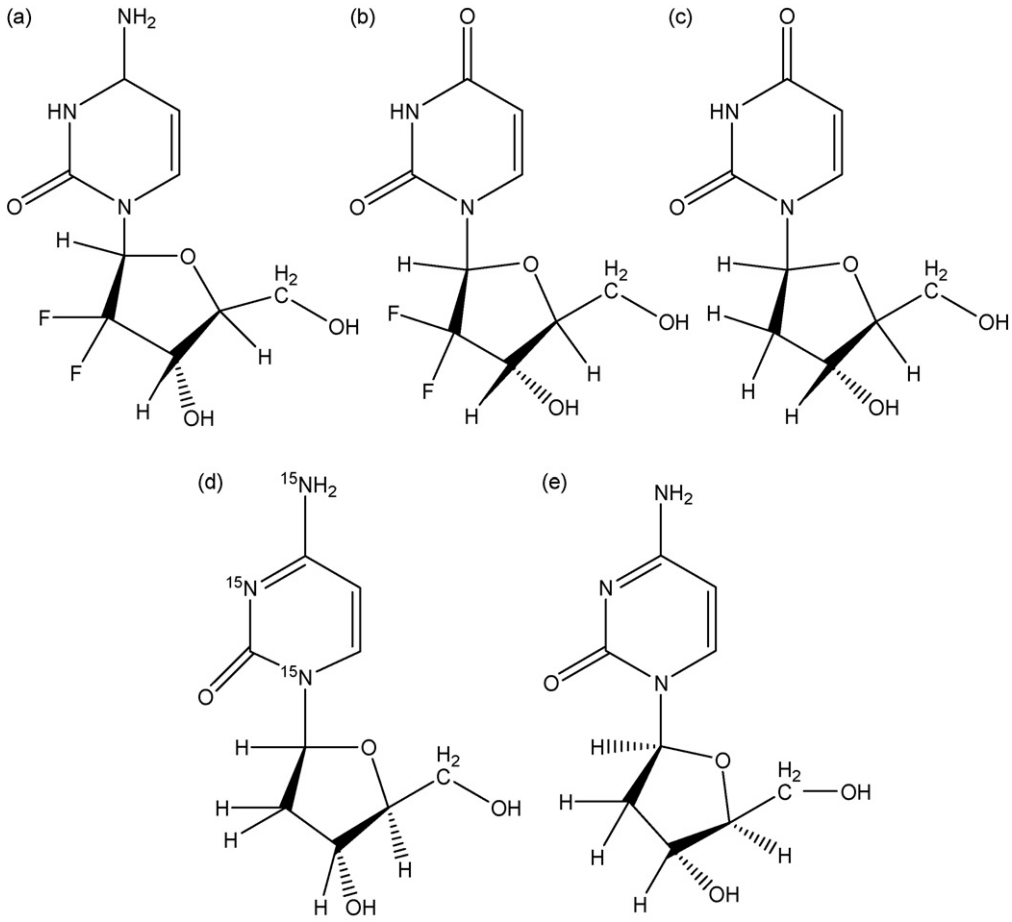

Fig. 1. Molecular structure of the compounds under investigation: (a) difluorodeoxycytidine (synonym gemcitabine, dFdC); (b) difluorodeoxyuridine (dFdU); (c) deoxyuridine (UdR); (d) $^{15}N_3$ deoxycytidine; (e) deoxycytidine (CdR).

Current HPLC methods reported in literature use UV detection and have a sensitivity range of between 0.1 and 50 μg/ml [13,15–17]. This proved to be insufficient for the detection of gemcitabine given as a 24 h infusions [13,18,19]. Mass spectrometric detection linked to HPLC is a growing field in pharmaceutical analysis due to its greater sensitivity and selectivity. An additional advantage is the faster analysis times commonly found with LC–MS/MS techniques. Two validated methods have been reported for the determination of dFdC in human matrix using tandem mass spectrometry with a LLOQ of 0.019 μM [20,21]. However, one method was validated on urine and proved to be not useful for human plasma [20], while the other required solid phase extraction and gradient chromatography which proved to be unworkable for tissue extraction [21]. Hence we developed a sensitive, selective and rapid method using LC–MS/MS techniques for the determination of dFdC and CdR in human plasma and tissue using isocratic conditions.

2. Materials and methods

2.1. Materials

Analytical grade solvents such as acetonitrile, formic acid, ethyl acetate and iso-propanol were supplied by Merck & Co. (distributed by VWR, Amsterdam, The Netherlands). HPLC grade water was supplied via a MilliQ water purification system (Millipore, Amsterdam, The Netherlands) purified at 18 MΩ into glass using a QPAK2 cartridge and a UF cartridge directly into glass containers.

Reference standards for difluorodeoxycytidine (dFdC; gemcitabine) and its major metabolite difluorodeoxyuridine (dFdU), were provided by Lilly Research (Eli Lilly & Co., Indiana, USA). 2′-Deoxycytidine and 2′-deoxyuridine (UdR) were obtained commercially from Sigma–Aldrich (Zwijndrecht, The Netherlands); $^{15}N_3$ deoxycytidine was supplied by Cambridge Isotope Laboratories (distributed by CK Gas Products Ltd., Hook, UK) and tetrahydrouridine was obtained from Calbiochem (distributed by VWR, Amsterdam, The Netherlands).

2.2. Equipment

Freeze drying was performed with a Christ bench top freeze drier (Salm and Kipp, Breukelen, The Netherlands) at −85 °C. Chromatography was conducted using a Perkin-Elmer (Wellesley, USA) series 200 HPLC system coupled with an Applied Biosciences (Foster City, USA) SCIEX API 3000 mass spectrometer for detection. The interface between the HPLC and detection systems was an atmospheric pressure chemical

Table 1
Optimized parameters for dFdC, CdR and UdR as determined by infusion of 1 μg/ml solution of each compound dissolved in 50 mM formic acid:acetonitrile (9:1, v/v) into the mass spectrometer via a turbo ion spray source, flow rate = 10 μl/min

Compound	DP	FP	EP	P	CXP
dFdC	28	250	10	23	20
CdR	31	237	10	14	20
UdR	24	268	10	21	20
$^{15}N_3$ CdR	31	237	10	14	20

ionization (APCI) source. The injection system of the Perkin-Elmer series 200 HPLC was fitted with a 50 μl PEEK sample loop and all tubing post injection was 0.05 mm PEEK. All volumetric transfers were performed with in-house calibrated Eppendorf pipettes in sterile 1.5 ml Eppendorf polypropylene screw cap tubes. Software used for data acquisition and integration was Analyst version 1.1 from Applied Biosciences upgraded with patches specific for Perkin-Elmer LC system series 200 autosampler and pump.

2.3. Analytical procedure

2.3.1. Mass spectrometry optimization

The optimized APCI conditions were as follows: nebulising gas flow 11 l/min, curtain gas flow 9 l/min, collision activated dissociation gas flow (CAD) 4 l/min, nebulisier current 3.0, probe temperature 425 °C. Compound specific parameters such as declustering potential (DP); focusing potential (FP); entrance potential (EP); collision cell entrance potential (CE); collision cell exit potential (CXP) were optimized from a 10 μg/ml stock solution of each individual component (Table 1). The mass spectrometry conditions of each compound were determined by an infusion at 0.4 ml/h into a turbospray ionization source at room temperature using a variety of different solvent conditions, the solvent with the best response was selected for further optimization. A period of 5 min was allowed for equilibration before spectra were collected over the positive Q1 range of 50–2000 *m/z* for 3 s. Molecular ions were determined for fragmentation over a positive Q3 range of 50–300 *m/z* for 3 s. Quantitation was developed in positive MRM (multi reaction monitoring) mode by the monitoring of the determined transition pairs of *m/z* 264 (molecular ion)/112 (major fragment ion) for dFdC, *m/z* 228 (molecular ion)/112 (major fragment ion) for CdR, *m/z* 229 (molecular ion)/113 (major fragment ion) for UdR and *m/z* 231 (molecular ion)/115 (major fragment ion) for $^{15}N_3$ CdR. The DP, FP, CE and CXP were optimized for maximum signal response of each of the MRM transition pairs.

2.3.2. Chromatographic conditions

Chromatographic separation was performed on two-spherisorb phenyl econosphere glass columns (3.0 mm × 100 mm; 5 μm) fitted in series with a zero dead volume holder supplied by Chrompak (distributed by Varian, The Netherlands). The column was maintained at room temperature and isocratically eluted with a mobile phase consisting of 50 mM formic acid:acetonitrile (9:1, v/v). Mobile phase was filtered through a 0.2 μm pore nylon membrane filter and degassed by ultrasonication at 4 °C. The column protection was supplied by a Chrompak C18 cartridge, 50 μm. Flow rate was set at 1.0 ml/min and the peaks of interest eluted within 5 min after an injection of between 4 and 40 μl.

2.3.3. Sample collection

All standard/sample collection and preparation were performed on ice. Whole blood samples were taken from volunteers into either heparin or EDTA tubes directly spiked with tetrahydrouridine (25 μl of 10 mg/ml) to prevent deamination of dFdC and CdR. Plasma was prepared by centrifugation at 4 °C and stored at −20 °C until required. Tissue samples were acquired at the surgical site and immediately placed in liquid nitrogen and stored at −80 °C in metal containers until analysis.

2.3.4. Preparation of plasma standards

Stock solutions of dFdC, dFdU, CdR, UdR and $^{15}N_3$ CdR were accurately prepared in methanol at a concentration of approximately 1 mg/ml. Purity and weight variations were adjusted by diluting approximately 1:10 to give a accurate stock solution of 0.1 mg/ml. Subsequent dilutions of dFdC, CdR and UdR stock solutions were prepared to give standard combined calibration solutions of 10, 4, 2, 1, 0.4, 0.2, 0.1, 0.04 and 0.02 ng/μl. 10 μl of each dilution was added to 200 μl of control plasma for standard preparation in a 1.5 ml Eppendorf tube. $^{15}N_3$ CdR was used as an isotopic internal standard at a concentration of 100 μg/ml; 10 μl was aliquoted per tube.

2.3.5. Plasma sample preparation

10 μl of the internal standard dilution was added to a 1.5 ml screw cap Eppendorf tube and 200 μl of sample plasma aliquoted into each. Samples and standard preparations were vortexed briefly prior to addition of 1 ml isopropyl alcohol followed by further vortex mixing. Samples were allowed to stand on ice for 5 min before vortexing again and subsequent centrifugation at 4 °C/14,000 rpm for 5 min. The supernatant was transferred to a new 1.5 ml screw cap Eppendorf tube and frozen in liquid nitrogen. All frozen samples were freeze dried overnight until all liquid had been removed.

The dried samples were reconstituted in 1 ml of ethyl acetate and vortex mixed thoroughly. After a brief centrifugation at 14,000 rpm/4 °C the ethyl acetate was transferred to a clean 1.5 ml Eppendorf tube containing 100 μl of purified water. Each tube was thoroughly vortex mixed followed by centrifugation at 4 °C/14,000 rpm. The aqueous layer was then carefully transferred into a 200 μl HPLC injection vial insert.

2.3.6. Human tissue preparation

Prior to the extraction of tissue all containers and mill equipment were cleaned with water/absolute alcohol before being placed in liquid nitrogen. Approximately 20–50 mg of frozen tissue was accurately transferred to a sealed pre-cooled

container after which the frozen material was quickly pulverized using a vibrational mill [22]. The pulverized material was then transferred to pre-weighed 2 ml eppendorf tube. One millilitre of an internal standard solution prepared isopropyl alcohol (100 μg/ml; chilled to 4 °C prior to use) was accurately added. Each sample was vortex mixed and centrifuged at 14,000 rpm/4 °C before the supernatant was transferred to a clean vial. Each vial was frozen in liquid nitrogen and freeze dried overnight. The dried samples processed further as described for plasma above.

2.3.7. Data handling and calculations

A calibration curve for each analyte was determined using linear least square analysis with $1/x$, $1/x^2$ and without weighting. Parameters used to determine the validity of the linearity were the deviation of the slope, the accuracy of the fit of the line and the percentage difference of the determined concentration from the theoretical. Recovery was determined as the calculated concentration from the line of best fit/the theoretical concentration.

2.3.8. Validation procedure

Selectivity was determined as the ability of the analytical method to differentiate and quantify an analyte in the presence of other related components and from individual components of the sample matrix. The precision, accuracy and robustness were determined from the variation of duplicate control linearity's analyzed on seven different days. The recovery of the extraction procedure was calculated by comparing the peak areas of each standard concentration against equivalent absolute standard dilutions. Limit of detection was determined by successive standard dilutions calculating the signal to noise ratio, the limit set for detection was a signal/noise ratio of 3.

2.4. Pharmacokinetic protocol

Plasma samples were obtained from patients enrolled into a phase I trial performed to determine the feasibility, side effects and the maximum tolerated dose of gemcitabine given as a prolonged infusion in the hepatic artery. A comparison was made between the pharmacokinetics of gemcitabine during hepatic arterial infusion with intravenous infusion in each patient. Dosing was started at 75 mg/m^2/24 h and escalated to 180 mg/m^2/24 h during the course of the study. Blood samples were collected in all patients during day 1 and day 8 treatments in heparinized tubes containing tetrahydrouridine (25 μg/ml) in order to inhibit degradation of dFdC to dFdU. Plasma was separated by centrifugation and stored in 2 ml aliquots at −20 °C. Sampling schedule was conducted in the following fashion 0, 2, 4, 24, 24.5, 26 and 28 h.

2.5. Tissue investigation

Tissues were obtained from a study to determine whether dFdC would be taken up by glioblastoma. dFdC was administered just before surgery or during anesthesia to 10 patients with recurrent glioblastoma. Tumor samples were obtained between 2 and 4 h after administration, frozen immediately and stored at −80 °C.

3. Results and discussion

3.1. Mass spectra

Fig. 2 shows a full spectrum scan of dFdC across the range 50–2000 *m/z*. A clear response is observed at 264.1 *m/z* corresponding to the molecular ion, $[M+H]^+$. No adducts were observed corresponding to $[2M+H]^+$ or $[M+Na]^+$ or $[M+K]^+$. Fragments were observed in the Q1 scan at 115.1, 74.1 and 56.3 *m/z* that were not related to the solvent. The 115.1 and 56.3 *m/z* fragments were related to the break up of the ring structure in the ionization source but do not represent a major source of error, being relatively low in comparison to the 264.1 *m/z* response. The 74.1 *m/z* response was very intense and could be related to the break down of the sugar ring structure. The resulting fragment would contain a readily ionizable center and would theoretically give a better ion signal than the molecular ion. The ion was not considered to be significant in the objective of the determination of response 264 *m/z*. Since the sugar component of the molecule is a common feature of both compounds being analyzed and of other molecules that theoretically could be in the plasma extract it was not considered further.

The primary fragment observed in the fragmentation of the 264 *m/z* product ion was at 112 *m/z* which corresponded to the loss of the ribose unit (Fig. 2, insert). Significantly smaller fragments were observed at 79 and 95 *m/z* corresponding to rearrangements in the primary fragment (112) resulting in the loss of oxygen and nitrogen groups (Fig. 3). MRM parameters were optimized on the 264/112 transition.

Fig. 4 shows a full spectrum scan of CdR and $^{15}N_3$ CdR. A clear response for CdR was observed at 228.5 *m/z* while $^{15}N_3$ CdR was observed at 231.3 *m/z*. No adducts were observed corresponding to $[2M+H]^+$ or $[M+Na]^+$ or $[M+K]^+$ for either compound. Signals were maximized for the product ions 228 and 231, respectively. Fragments not related to the solvent were observed in the Q1 scan for CdR at 112.3, 160.2 and 183.3 *m/z*, but $^{15}N_3$ CdR at 115.3, 122.5 and 102.3 *m/z*. The 160.2 response was related to the loss of cysteine from the CdR ring structure, whereas, 112.2 and 115.3 represent the loss of the ribose unit from the respective molecules CdR and $^{15}N_3$ CdR. For CdR 183.3 *m/z* was the major observable Q1 fragment and could be related to the loss of CH_2CHOH from the ribose ring structure, this was not observed for $^{15}N_3$ CdR. For $^{15}N_3$ CdR significantly smaller Q1 fragments were observed at 99 *m/z* and 145 *m/z* corresponding to ions formed from the detached ribose unit.

For MRM analysis the primary ions from the Q1 scan were fragmented for screening in a Q3 spectrum. Primary CdR and $^{15}N_3$ CdR fragments observed were 112 and 115 as described above. MRM parameters were optimized for CdR on the 228/112 transition pair and $^{15}N_3$ CdR 231/115, respectively

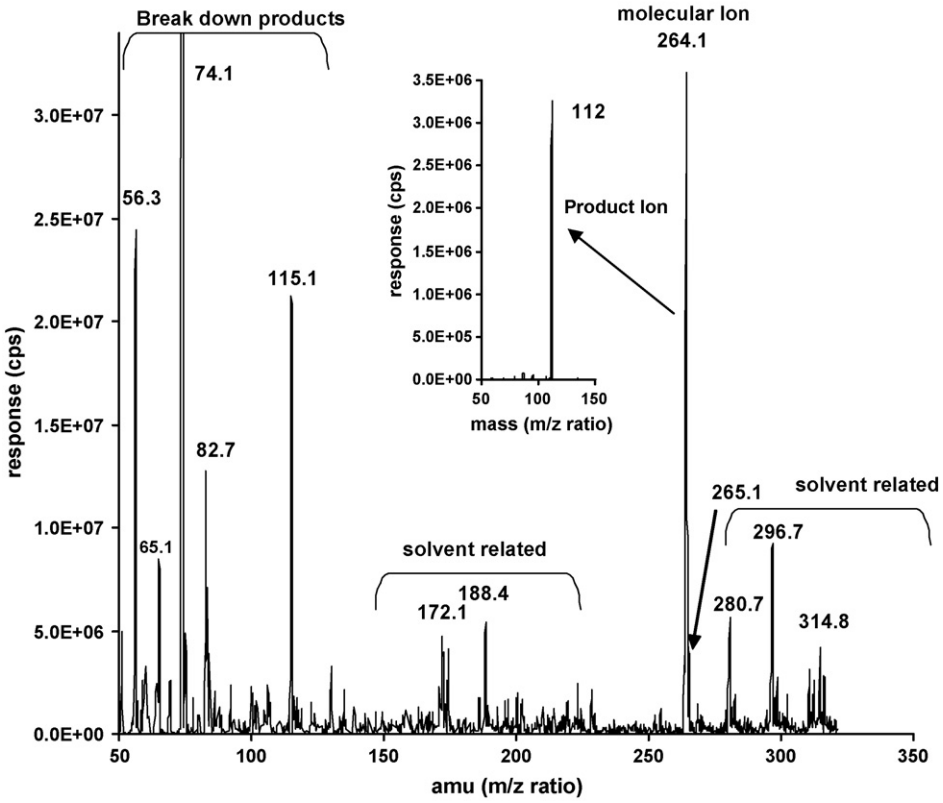

Fig. 2. The 50–350 *m/z* segment from a full spectrum scan (50–2000 *m/z*) of dFdC in 90% 50 mM formic acid:10% acetonitrile with the 50–150 *m/z* product ion spectrum (insert) of the $[M+H]^+$ ion (264.1 *m/z*).

(Fig. 4, insert). UdR and dFdU were optimized in a similar fashion as CdR and dFdC establishing optimized transition pairs of 229/113 *m/z* and 265/113 *m/z*, respectively (data not shown).

3.2. Activity

Each compound was infused directly into the MS detection system and all MRM transitions were monitored. A % non-specific baseline was calculated based on the total ion count observed for dFdC, dFdU, CdR, UdR and $^{15}N_3$ CdR. No evidence of a response could be observed for dFdC following infusion of CdR, or $^{15}N_3$ CdR. A minimal to no signal was observed for CdR after infusion with $^{15}N_3$ CdR. THU, dFdU and UdR were also investigated and no interference could be observed with CdR and dFdC transitions pairs (Table 2). However, in reverse infusions, CdR and dFdC demonstrated a $[M+1]^+$ response for UdR and dFdU, respectively. To determine UdR and dFdU in the same analysis as CdR and dFdC, a complete chromatographic separation is necessary to avoid "crosstalk" contamination. In the mass spectrometry optimization the optimal solvent conditions to maximize sensitivity for CdR and dFdC consisted of 50 mM formic acid and acetonitrile. Chromatography was established using conditions of 9:1 (v/v) aqueous: solvent ratio with CdR and dFdC co-eluting at approximately 2.3 min (Fig. 5). Under these conditions UdR and dFdU co-eluted at approximately 1.7 min. Note also that in blank plasma endogenous CdR gives a signal approximately three to four times the baseline.

3.3. Limit of detection

The detection limit was set for each compound as the lowest concentration that had a signal to noise ratio of 3 or greater. Signal to noise ratio (S/N) was calculated as being the peak intensity divided by the average of the noise preceding the peak elution under optimized chromatographic conditions. dFdC gave a signal as low as 0.5 ng/ml but with a signal to noise ratio of less than 3, hence the detection limit was set at 1 ng/ml, which has a S/N ratio of 3.5. CdR gave a slightly less sensitive signal where the detection limit was set to 1.5 ng/ml. UdR was relatively less sensitive compared to CdR and dFdC when extracted from plasma alone. However, when extracted from plasma containing THU and dFdU, UdR was subject to intense interference limiting the quantitative determination of this component.

The limit of quantitation (LOQ) was set at 0.019 μM for dFdC, and 0.022 μM for CdR. The values are well below the detection limits reported for HPLC/UV assays from both ourselves and others (Table 3). The described extraction methods used different but more traditional extraction techniques, however, we observed ion suppression and chromatographic interference from these forms of extractions whereas, the use of freeze drying in conjunction with a back-extraction approach

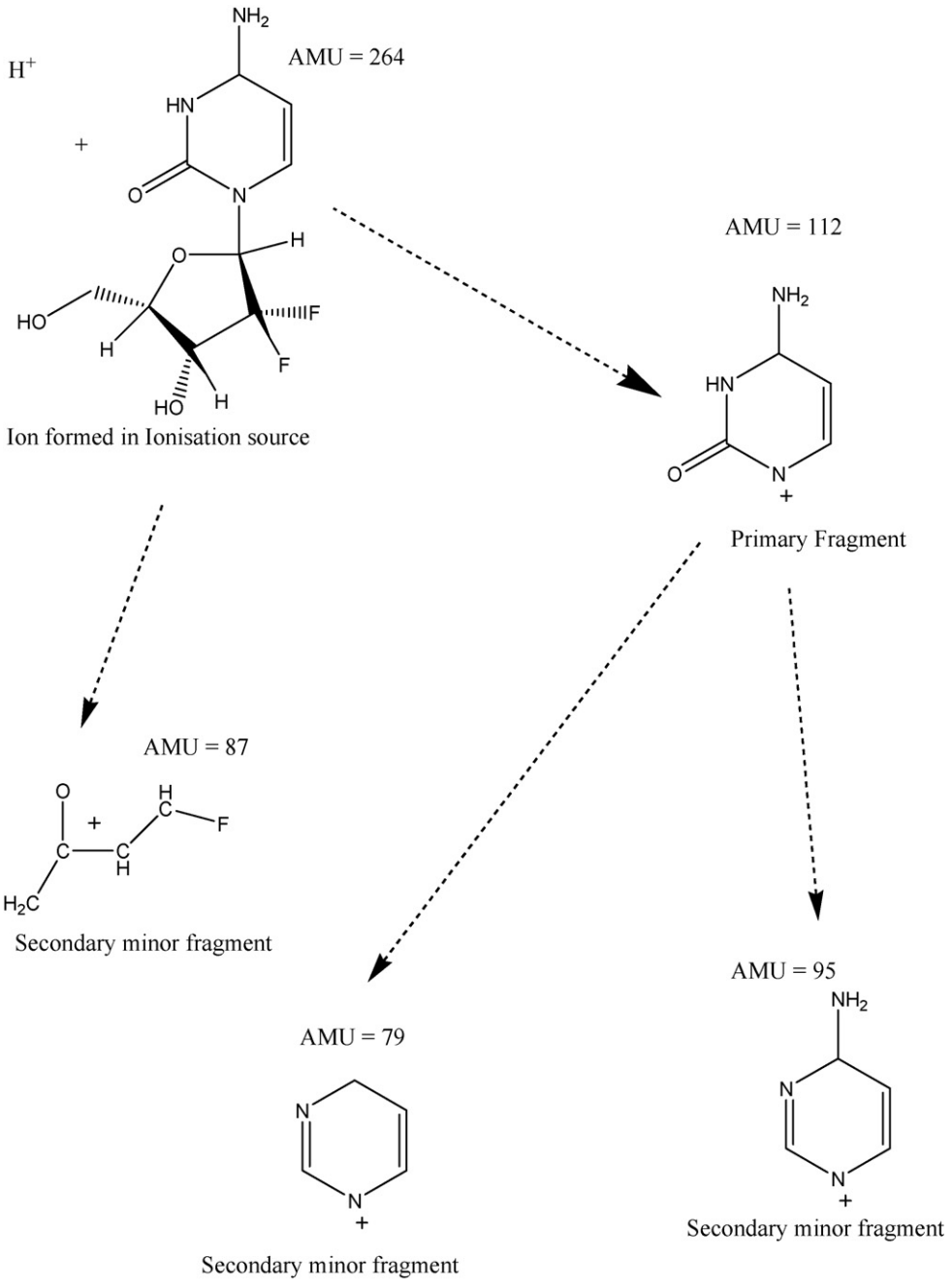

Fig. 3. Suspected fragmentation pattern for dFdC.

to water potentially led to more favorable results. The observed sensitivity was similar to that reported by Xu et al. [21] but the simplicity of the extraction and shorter chromatographic time represented an added benefit. The use of MRM techniques provided greater selectivity for each component but limited the maximum detectable.

The lower sensitivity to dFdU presented no problems since the plasma concentrations of dFdU would always be greater than the upper limit to the mass sensitivity. dFdU can easily be measured with a standard HPLC-UV techniques, detectable well after dFdC administration [23]. The lack of sensitivity to UdR was directly attributable to the levels of dFdU present in the concentrated sample.

3.4. Recovery, precision and accuracy

The results for dFdC and CdR extracted from human plasma are shown in Table 4, whereas, tissue extractions were compared to neat standard regressions. This was due to the limited tumor tissue available (50–150 mg); hence, the preparation of standard regressions in sample matrix was impossible. However, the plasma recovery was calculated to be nearly 100% for all components using this extraction technique and a similar recovery was assumed for tissue with given limitations. Precision and accuracy for both compounds were within ±15% of expected, which, conforms to known regulatory specifications of analytical methodology (Table 4). UdR did not conform to the ±15%

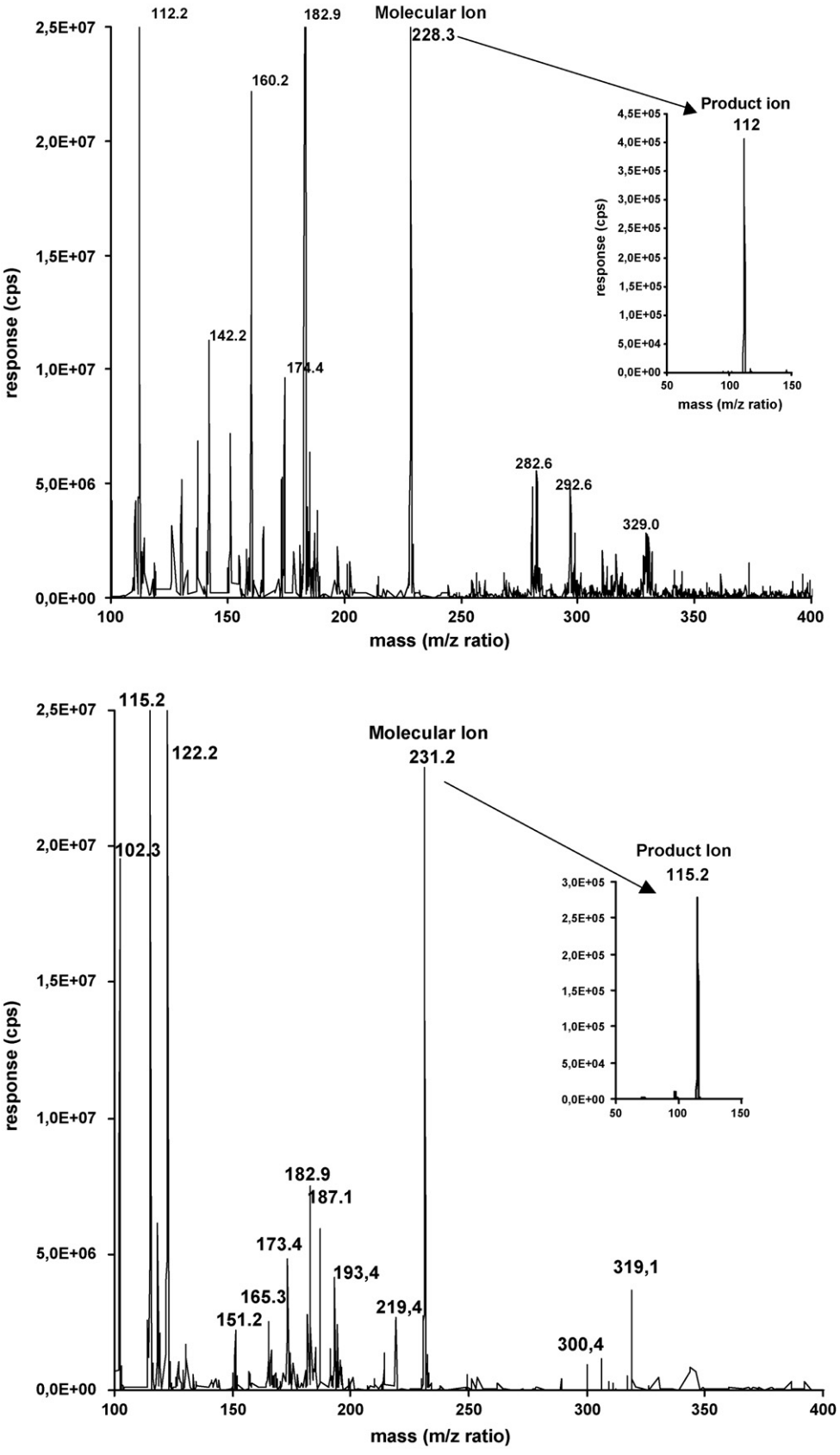

Fig. 4. The 50–350 *m/z* segment from a full spectrum scan (50–2000 *m/z*) of CdR and $^{15}N_3$ CdR in 90% 50 mM formic acid:10% acetonitrile. The molecular ions ($[M+H]^+$) for CdR and $^{15}N_3$ CdR can be observed at 228 and 231 *m/z*, respectively.

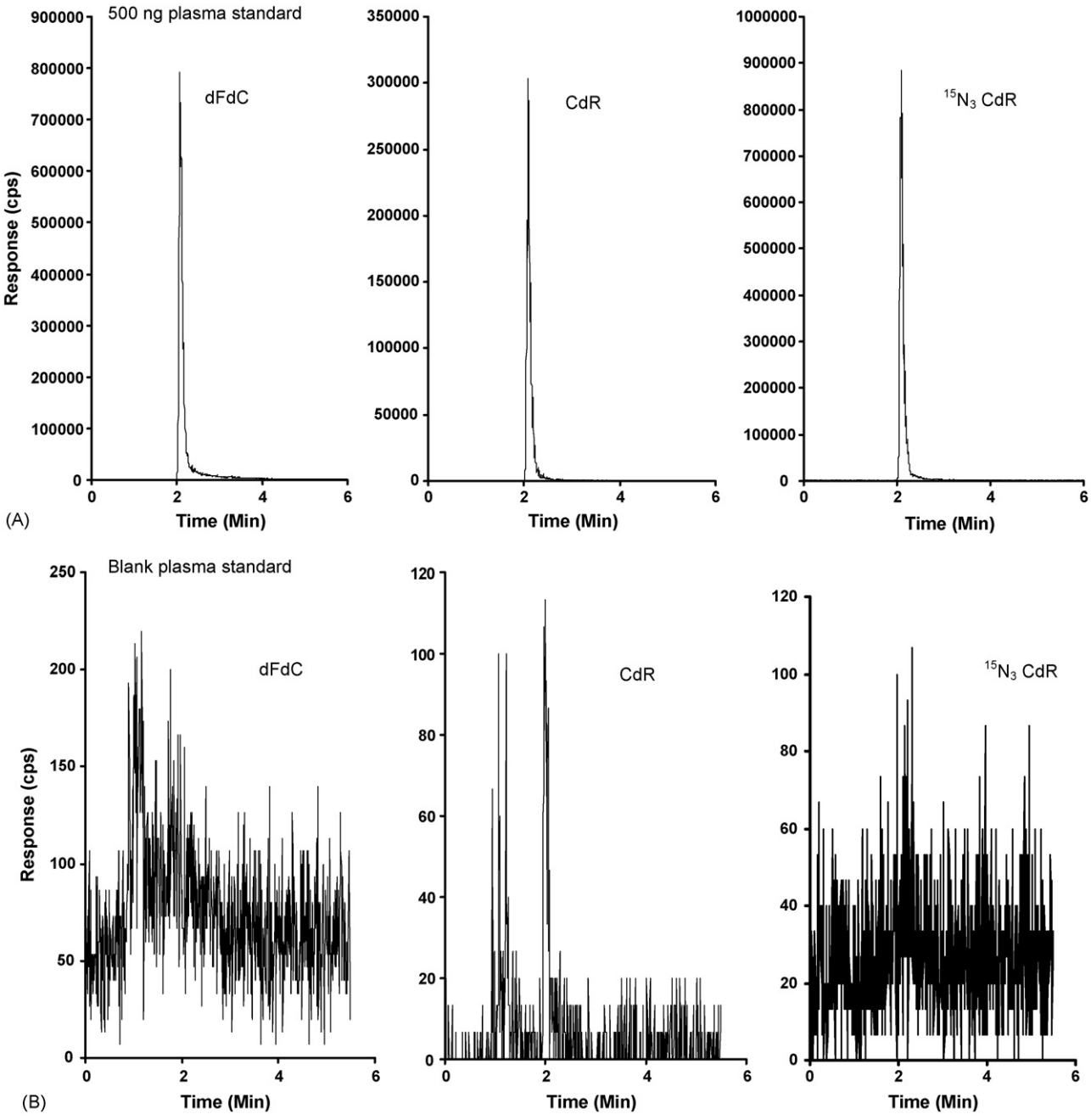

Fig. 5. Plasma extracted 500 ng/ml standard (A) and plasma extracted blank sample (B) for dFdC and CdR using chromatographic conditions of 9:1 (v/v) 50 mM formic acid:acetonitrile at 1 ml/min. Ionization source was a turbo ion spray set at 425 °C and an ion voltage of 3.5 kV.

guideline specification, chromatographic separation was poor and subject to the influence of co-eluting negative peaks. Interference was traced to the presence of dFdU and THU extracted from the plasma. Quantitative determination of both UdR and dFdU was not realistically possible under these circumstances.

3.5. Linearity

Each component has a different response to mass spectrometric detection and the line of best fit was determined by comparing the least squared linear regression using either using weighting factors ($1/x$, $1/x^2$) or no weighting. The situation of the best linearity was calculated as the regression which used the lowest weighting group and gave the broadest range of accuracy. The difference between using area or the peak height was also determined. dFdC showed good linearity under all weighting factors but best demonstrated linearity using $1/x$ weighting with area with an average slope of 1.051 ± 0.13 and intercept of -6.335 ± 7.17, regression coefficient was 0.9982. CdR also demonstrated good linearity with all weighting groups and the best regression was determined to be that which involved no weighting. Average regression data was calculated as slope = 0.988 ± 0.05, intercept = 2.638 ± 3.52 and a regression coefficient of 0.9996. UdR demonstrated good linearity

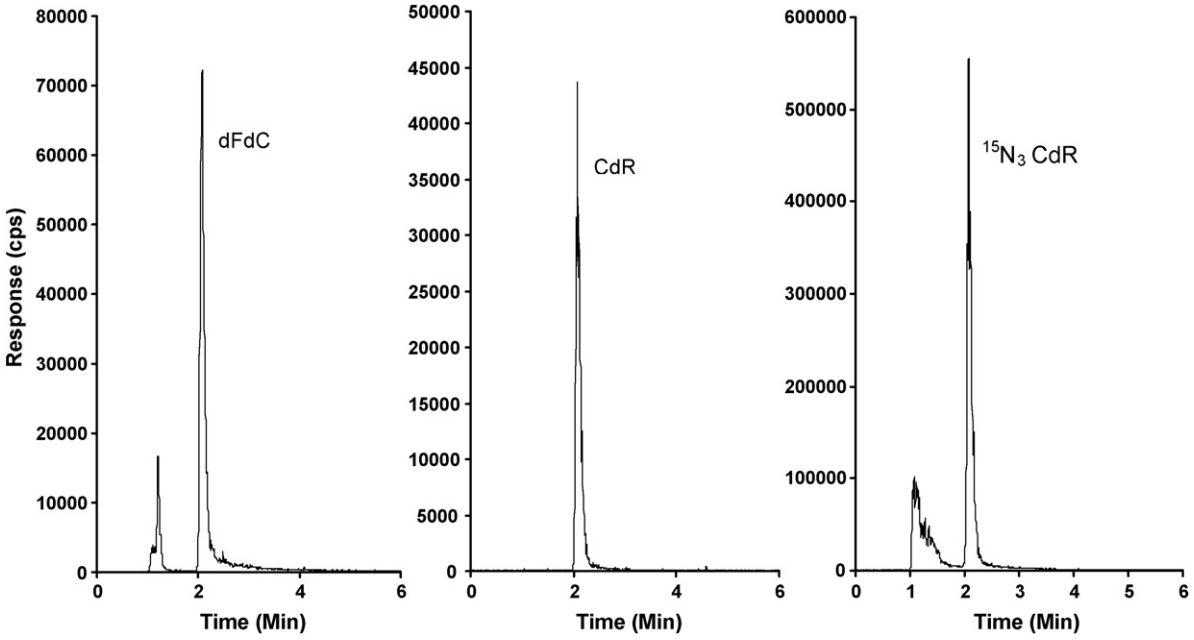

Fig. 6. Chromatogram of a tissue extracted sample demonstrating the sensitivity and selectivity of the developed method. The sample shown is for Glioblastoma tissue taken from a male subject 1.5 h after infusion with 1000 mg/m^2 dFdC on a 30 min schedule. Relative concentrations found were 0.64 nmol/g tissue for dFdC and 0.69 nmol/g tissue for CdR.

above 20 ng/ml with $1/x^2$ weighting when extracted from blank plasma, however, linearity was significantly adversely affected when dFdU was included in the standard extract. Chromatography was subject to disruption at baseline (negative peaks) masking UdR detection below 50–100 ng/ml. Considering the required sensitivity for this component (1–10 ng/ml) this assay did not represent the necessary quantitative accuracy we required. Further work on UdR for quantitative analysis was performed using a different approach not reported here. It was not necessary to continue with dFdU quantitative analysis since an existing HPLC-UV method already provides sufficient sensitivity and accuracy for this component.

The linearity of the assay was comparable to other assays, either HPLC-UV or LC–MS, although the sensitivity for HPLC-UV assays have a higher LOQ of approximately 0.1–0.4 μM (Table 2). The limitation found for this assay was in the determination of UdR, although this did not affect the analysis of dFdC. Measured data for UdR indicated that plasma concentrations did not increase significantly whereas, CdR was observed to increase during dFdC infusion (data not shown). However, the assay would be suitable for the detection of UdR in plasma of patients or animals treated with thymidylate synthase inhibitors, where the UdR sensitivity is increased significantly due to the removal of the dFdU interference. This assay has demonstrated a higher sensitivity to most reported HPLC assays, which require more extensive extraction procedures.

3.6. Tissue

Fig. 6 shows a tissue extraction of a patient treated with dFdC obtained from a separate clinical trial. The tissue sample was part of a collection stored at −80 °C for 2–3 years prior to the development of this method, indicating the stability of the samples under these storage conditions. Original HPLC-UV analysis did not show any dFdC content; however, the chromatogram observed is clear of interference and shows significant dFdC and CdR levels to be present.

3.7. Plasma

The method was initially used to determine low levels of dFdC in plasma from patients receiving a 24 h infusion. The pharmacokinetic results of that investigation will be reported elsewhere. However, Fig. 7 illustrates the applicability of this method with a concentration versus time profile from one patient given 180 mg/m^2. CdR was also determined over the course of the pharmacokinetic investigation and surprisingly, following gemcitabine treatment CdR plasma levels increased over time

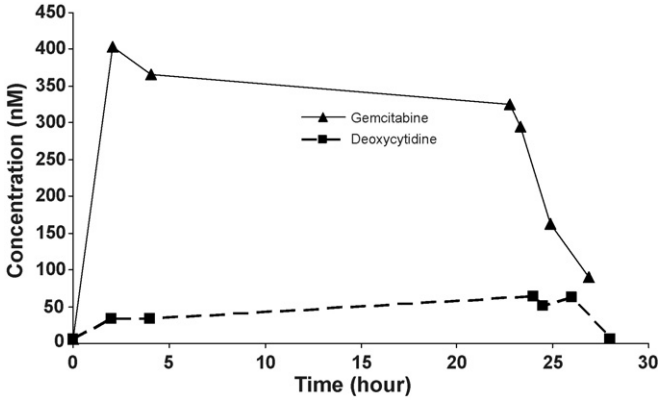

Fig. 7. Concentration vs. time profile from one patient treated with 180 mg/m^2 dFdC infused intravenously over a 24-h period.

Table 2
Comparison of the most recently published methods for the determination of gemcitabine and their relative sensitivities for detection in plasma extracts

Reference	Year	μM LOD (min–max)	Detection	Wavelength (nm)	Column	Mobile phase	Isocratic/gradient
[18]	2003	0.5–150	UV/diode array	269 dFdC 258 dFdU	Phenomenex C18, 150 × 4.6, 5 μm	50 mM AmA (pH5.0):MeOH	Gradient
[24]	2003	0.3–76	UV	272 dFdC	Hypersil 250 × 4.6, 5 μm	20 mM Phos B (pH 3.1) MeOH + 10 mM sodium heptane sulphonic acid	Isocratic
[25]	2003	0.76–190	UV	272 dFdC	Nucleosil 250 × 4.0, 5 μm	MeOH:cyclohexane:DCM	Isocratic
[17]	1996	0.19–10.8	UV	272 dFdC	Econosphere NH2 250 × 4.6, 5 μm	Cyclohexane:DCM; MeOH; water; glacial acetic acid; TEA	Isocratic
[19]	2004	0.76–200	UV/diode array	268 dFdC	C18 250 × 4.6, 5 μm	40 mM AmA (pH5.5):acetonitrile	Isocratic
[21]	2004	0.019–3.8	LC–MS	Single ion monitoring 262.0	YMC ODS-AQ 150 × 2.0, 5 μm	5 mM AmA (pH6.8):MeOH	Gradient
[20]	2004	0.76–19.0 (note for urine only)	LC–MS/MS	Transition pair 264.0/112.0	BDS C8, 150 × 4.6, 5 μm	0.1% formic acid:acetonitrile	Gradient
Current paper	2006	0.019–3.8	LC–MS/MS	Transition pair 264.0/112.0	Econosphere NH2 200 × 3.1, 5 μm	50 mM formic acid:acetonitrile	Isocratic

AmA, ammonium acetate; Phos B, phosphate buffer; MeOH, methanol; DCM, 1,2-dichloromethane; TEA, triethylamine.

Table 3
Percentage relative ion counts observed for 264/112, 228/112 and 231/115 MRM transitions following infusion of neat standard solutions of dFdC, dFdU, CdR and UdR

Compounds MRM monitor				
	dFdC	CdR	UdR	$^{15}N_3$ CdR
dFdC	Baseline of $1.15e^5$ cps	0.02%	0.03%	0.1%
CdR	0.07%	Baseline of $2.8e^5$ cps	0.4%	0.05%
UdR	0.03%	0.4%	Baseline of $3.8e^4$ cps	0.06%
$^{15}N_3$ CdR	0.02%	0.12%	0.06%	Baseline of $3.8e^5$ cps
dFdU	0.13%	0.004%	0.07%	0.03%

Table 4
Recovery, precision and accuracy of the LC–MS/MS assay for gemcitabine and deoxycytidine extracted from human plasma

	Spiked concentration (ng/ml)	Calculated concentration (ng/ml) CV	% Recovery
dFdC	500	522.8 ± 4.6	90.1
	100	93.8 ± 6.2	
	10	11.0 ± 10.2	
CdR	500	500.3 ± 0.1	99.2
	100	102.4 ± 2.4	
	10	10.5 ± 4.6	
UdR	1000	1008.1 ± 0.8	100.4
	100	102.1 ± 2.1	
	50	49.2 ± 1.7	

until the end of infusion (24 h) (Fig. 7). The assay has subsequently been applied to the determination of systemic exposure to locally administered dFdC to the liver with a hepatic artery infusion.

4. Conclusion

The developed method was demonstrated to be selective and reliable for plasma and tissue extractions with reference to dFdC and CdR. In comparison to traditional HPLC-UV methods the assay demonstrates a several fold (10–20) increase in sensitivity, while the selectivity of the LC–MS/MS technique enables the measurement of dFdC in various types of tissue without interference. The UdR response was subject to interference that was linked to the high concentration of dFdU and THU present in each sample. This prevented the determination of UdR with any reliability; however, the basic method was shown to be reliable and can be used for the fast determination of low concentrations of UdR in plasma, provided dFdU or THU is not present.

The developed method has demonstrated sufficient sensitivity to detect low levels of gemcitabine and deoxycytidine in human plasma and tissue. This procedure can be applied retrospectively to clinical samples with previously undetectable gemcitabine levels stored under correct conditions as well as provide a fast, sensitive method of detection for future clinical investigations.

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